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ORIGINAL ARTICLE

RNA recombination in Hepatitis delta virus: Identification of a novel naturally occurring recombinant

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Abstract *Background/Purpose:* Hepatitis delta virus (HDV) is the only animal RNA virus that has an unbranched rod-like genome with ribozyme activity. It replicates in the nucleus by host RNA polymerase via a rolling circle mechanism. Similar to many RNA viruses encoding their own RNA-dependent RNA polymerases, homologous recombination of HDV occurs in mixed-genotype infections and in cultured cells cotransfected with two HDV sequences, as demonstrated by molecular analyses.
Methods: Among 237 published complete genomic sequences, 34 sequences were reported from the small and isolated Miyako Island, Japan, and belonged to the Asia-specific genotypes, HDV-2 and HDV-4 (the majority of them belonged to the known Miyako Island-specific subgroup, HDV-4M). We investigated the presence of naturally occurring HDV recombinant in Miyako Island using phylogenetic and recombination analyses.

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Results: We identified a two-switch HDV-4/4M intersubtype recombinant with an unbranched rod-like RNA genome.
Conclusion: Our data suggest that RNA recombination plays an important role in the rapid evolution of HDV, allowing the production of new HDV strains with correct genomic structures.

Introduction

Hepatitis delta virus (HDV) was discovered by Rizzetto et al[1] in 1977 while investigating a group of patients with a severe form of chronic hepatitis B. HDV is a small, defective RNA virus with a negative-stranded, circular RNA genome of about 1.7 kb in length.[2,3] The HDV virion, which is 36 nm in diameter, is enveloped by surface proteins from its helper hepatitis B virus (HBV). Therefore, HDV propagates only in an individual who has coexisting HBV, due either to coinfection of the two viruses or superinfection of a chronic HBV carrier.[1,4] Superinfection is associated with a more threatening form of liver disease leading to rapid progression to cirrhosis and increased risk of hepatocellular carcinoma.[4] Globally, there are approximately 250 million HBV carriers, an estimated 15 million of whom have HDV infections.[4] HDV circular genome folds into an unbranched, rod-like structure with about 70% intramolecular base-pairing[2,3] and it has ribozyme activity.[5] HDV hijacks the host RNA polymerase to propagate the viral genome in the nucleus via a rolling-circle mechanism.[6,7] Three different HDV RNAs are detected within the cells: full-length genome, antigenome, and mRNA, which is ~900 nucleotides (nt) long and of antigenomic polarity.[6] It translates into the only known viral protein, the hepatitis delta antigen (HDAg), which has no polymerase activity. There are two forms of HDAg; the small one supports HDV RNA replication, while the large form is crucial for virion assembly.[8,9] During HDV RNA replication, some of HDV antigenomic RNAs are edited by a small form of adenosine deaminase acting on RNA 1, which changes adenosine to inosine specifically at amber/W site. The UAG amber termination codon of the small HDAg is thereby changed to the UGG tryptophan codon, and translation proceeds to produce large HDAg. Therefore, the N-terminal 195 amino acids of the two HDAgs are the same; the small one differs by only 19—20 amino acids at the C-terminus of the large HDAg.[10]

Nucleotide sequence analyses have initially classified HDV isolates into just three genotypes[11] and then into a total of eight clades.[12] They differ in their global distributions.[12] HDV-1 is prevalent worldwide. HDV-2 and -4 are exclusively Asian in origin.[13,14] HDV-3, and -5—8 are isolated from South America and African, respectively.[11,12] The HDV-4 sequences isolated from Miyako Island of Okinawa, Japan, include two clusters: one shows significant homology to HDV-4 (first isolated in Taiwan), while the other forms a subgroup that has been designated HDV-4M (M stands for Miyako).[15] Pathogenicity may vary among the HDV genotypes: HDV-1 has been associated with a broad spectrum of pathogenicity; HDV-2 causes milder forms of liver disease;[16] and HDV-3 has been associated with outbreaks of a severe form of fulminant hepatitis.[11] Furthermore, Miyako Island patients with HDV-4M reportedly show greater progressions to chronic hepatitis and cirrhosis compared with those with HDV-4.[15]

Similar to many RNA viruses, HDV exhibits high genetic heterogeneity. In addition to well-documented polymerase incorporation errors and RNA editing, RNA recombination, which may reflect the template-switching activities of host RNA polymerases acting on an atypical viral RNA template,[17] has been observed in mixed-genotype infections and in cultured cells cotransfected with two HDV sequences.[18—20] In addition to phylogenetic analysis, which provides a robust and informative test of the recombination hypothesis, many recombination analysis and detection methods have been developed. To provide an additional line of evidence for the significance of RNA recombination in HDV, we thereby used a strategy involving the computational analysis of complete genomes to better understand the occurrence of RNA recombination in nature. We herein collected published full-length HDV genomic sequences. Of them, 34 were from Miyako Island,[21,22] a relatively small and isolated geographic area where the virus is endemic and multiple genotypes have been identified. We used multiple phylogenetic and recombination analyses to observe a naturally occurring HDV intersubtype HDV-4/HDV-4M recombinant with an unbranched rod-like RNA structure.

Methods

Phylogenetic and recombination analyses

Phylogenetic analyses of the whole-genome alignment and all of the sub-datasets were performed with neighbor-joining (NJ) and maximum-likelihood methods under Kimura's two-parameter model.[23,24] A bootstrap test and reconstruction was done 1000 times to confirm the reliability of the phylogenetic trees. Trees were visualized using MEGA6.[41] If evidence for phylogenetic incongruence was apparent due to a change in the topological positions of specific sequences collected in the sub-datasets, the potential recombinants and their parental sequences were subjected to additional recombination analysis. We used the RDP software version 4,[25] which contains a series of recombination-detecting algorithms,[26—29] including BootScan, 3Seq, Chimera, and MaxChi. These methods also provided the breakpoints and *p* values. The outputs of these methods were compared. The major and minor

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parents were defined as those contributing the larger and smaller fractions of the recombinant sequence, respectively.[25] The predicted rod-like structures were generated using RNAstructure version 3.7.[30]

Results and discussion

Collection and analyses of HDV sequences

Relatively few cases of mixed-genotype infections have been reported,[18,31] complicating the direct investigation of HDV RNA recombination from natural infections. However, the recombination events of many viruses have been identified with the computational analysis of complete genomes. Here, we analyzed HDV RNA recombination using the full-length genomic sequences available for HDV. To identify potential naturally occurring HDV recombinants, we wanted to select a group of HDV sequences obtained from a relatively small and isolated geographic area where the virus is endemic, multiple genotypes have been identified, and numerous complete sequences are available. Since the first HDV RNA database, comprising 83 complete HDV sequences, was published in 2006,[32] numerous additional HDV clones have been isolated from around the world. Here, we downloaded 234 full-length HDV genomic sequences from GenBank. Among the collected sequences, 139 (~61%) belonged to the globally distributed HDV-1, while HDV-2–8 were represented by 16, eight, 37, 15, six, five, and five sequences, respectively. HDV-2 and -4 are exclusively found in Asia, and the latter has only been found in Taiwan and Miyako Island. Although chronic HDV infection is relatively rare on mainland Japan, it is endemic on the small and closed environment of Miyako Island.[33,34] Importantly, 31 HDV sequences (~13% of the total) had been isolated from Miyako Island. Therefore, Miyako Island was the only geographic area that met the above-mentioned criteria for detecting potential HDV recombinants by the computational analysis of complete genomes. These 31 sequences together with three additional Miyako sequences (cases 1–3, which have been published[22] but not submitted to GenBank) were subjected to phylogenetic analysis. A NJ phylogenetic tree was constructed using the complete HDV sequences of the 34 Miyako Island sequences and references sequences of Asian origin. One and five of the Miyako isolates clustered into HDV-2 and -4, respectively, while the remaining 28 formed HDV-4M (Figure 1). These data were consistent with a previous report indicating that majority of the Miyako isolates belonged to a subgroup of HDV-4.[15]

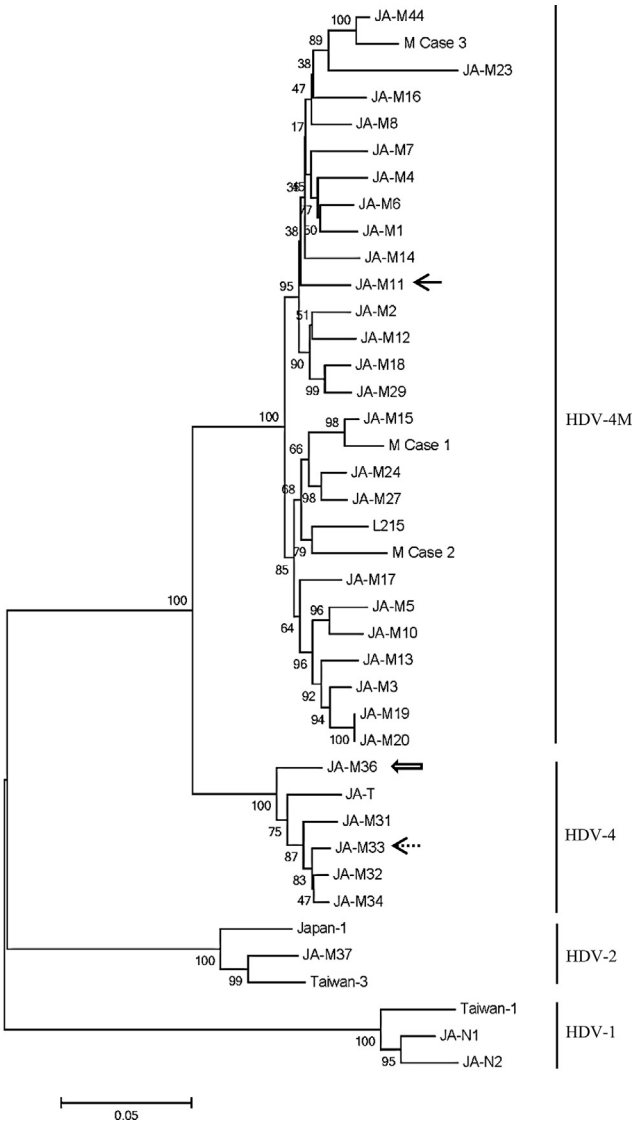

Figure 1. Neighbor-joining phylogenetic trees of full-length Hepatitis delta virus (HDV) sequences. The 34 HDV sequences isolated from Miyako Island were labeled as previous reports.[15,21,22] The GenBank accession numbers for L215 and JA-M1–JA-M37 are AB088679, AF309420, and AB118818–AB118846. The Miyako Island sequences were compared to Asian HDV reference sequences representing HDV-1, -2, and -4 (denoted on the right). The accession numbers of the HDV reference sequences were as follows: JA-T, AB118847; Japan-1, X60193; Taiwan-1, M92448; Taiwan-3, U19598; JA-N1, AB118848; and JA-N2, AB118849. Bootstrap resampling and reconstitution were carried out 1000 times. The numbers at the nodes indicate the bootstrapping values. The bar represents nucleotide substitutions per position.

Naturally occurring intersubtype recombinant in Miyako Island

Given that multiple HDV genotypes/subtypes have been isolated from the small Miyako Island, we then examined their published sequences for any potential naturally occurring HDV recombinant. We applied a strategy in which we compared the phylogenetic signals from four subdivided fragments of the full-length genomic sequence. Since the HDV genome is circular, we decided to use a region spanning nt 850–1300 (comprising ~$^1/_4$ of the HDV genome) as the first segment; this sequence, which is frequently used for HDV genotyping,[11,12] includes a region near the antigenomic cleavage site and the sequences encoding the C-terminus of HDAg. The four sub-datasets used herein contained subgenomic sequences spanning nt 850–1300 (Segment I), nt 1301–80 (Segment II), nt 81–470 (Segment III), and nt 471–849 (Segment IV). The former two segments were

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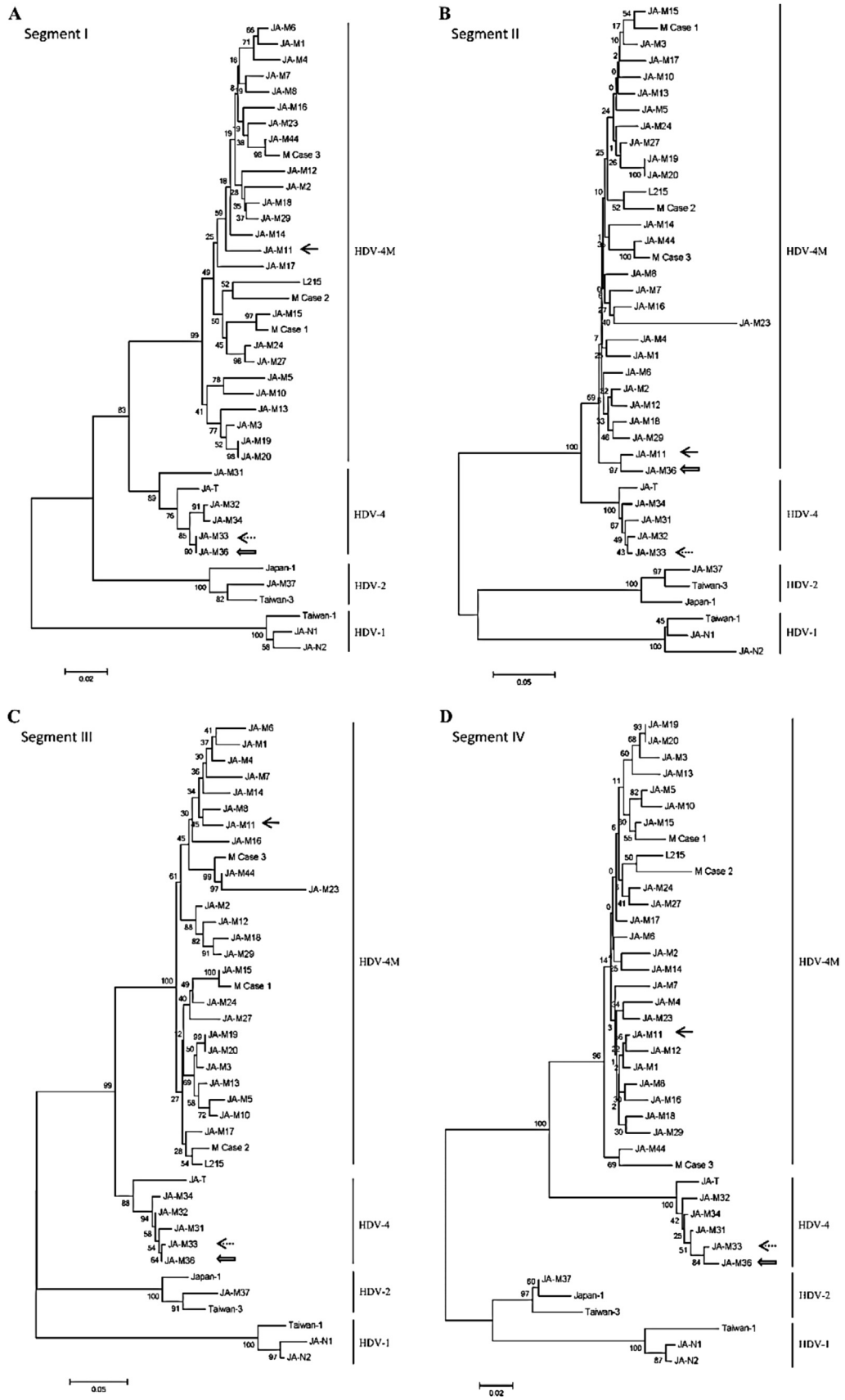

Figure 2. Neighbor-joining phylogenetic trees constructed for the Hepatitis delta virus (HDV) sequences using four subdomains. Trees A—D were inferred for the segments defined by nucleotides (nt) 850—1300, nt 1301—80, nt 81—470, and nt 471—849,

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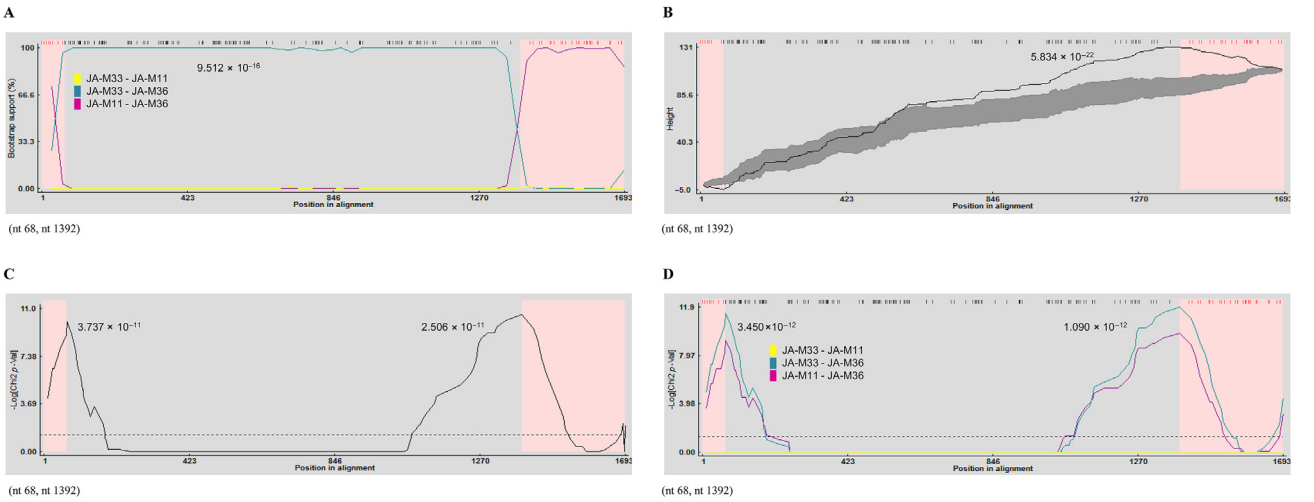

Figure 3. Analyses of the breakpoints of the novel recombinant JA-M36: (A) BootScan analyses of JA-M36 and its potential parents. The y-axis gives the percentage of permutated trees in a sliding 150-bp window with 30-bp steps. The arrows indicate the breakpoints; similarly, (B) 3Seq; (C) Chimera; and (D) MaxChi analyses were used to confirm JA-M36 is a recombinant. The *p* values and breakpoints are shown. Nt = nucleotides.

~450 bp long, while the latter two regions were obtained by equally dividing the remaining HDV sequences. Using these four sub-datasets, NJ phylogenetic trees were constructed. The phylogenetic trees for Segments I, III, and IV showed that JA-M36 and JA-M33 belonged to HDV-4, while JA-M11 was a HDV-4M isolate (Figures 2A, 2C, and 2D). These findings were consistent with the results obtained from the full-length tree (Figure 1). However, a different phylogenetic relationship was found when Segment II was subjected to analysis. JA-M36 clustered into HDV-4M with JA-M11 as the closest sequence (Figure 2B). The presence of discordant phylogenetic signals with strong bootstrapping support strongly suggested the existence of recombination. To further confirm these results, we performed maximum-likelihood phylogenetic analysis using the same sub-datasets. The results were compatible with those of the NJ phylogenetic trees shown in Figure 2 (data not shown). These data also strongly suggested that JA-M36, a HDV-4/4M intersubtype recombinant, was generated by at least two recombination events occurring between JA-M33 (major parent, HDV-4) and JA-M11 (minor parent, HDV-4M). Taken together, the results of these two different phylogenetic analyses indicated that, from among 34 Miyako isolates, we identified sequence JA-M36 as potentially being a mosaic structure assembled from JA-M33 and JA-M11.

Recombination analyses of HDV recombinant JA-M36

Four different methods of recombination detection were used to verify the results obtained from our phylogenetic analyses. The relationship of the recombinant with the major and minor parents was first visualized with BootScan, which confirmed our findings and further indicated that the breakpoints were at nt 68 and nt 1392 (Figure 3A). The former is adjacent to the potential RNA promoter region[35] and the latter falls within the HDAg open reading frame (ORF). 3Seq, Chimera, and MaxChi analyses were then used as alternative approaches for detecting recombination (Figures 3B–D). Although these methods are based on different rationales, they all detected JA-M36 as a recombination between JA-M33 and JA-M11 with significant *p* values and identified the same novel breakpoints in JA-M36 (Figure 3). Visual inspection of the nucleotide sequence alignment covering nt 1573–190 and nt 1240–1539 further supported the breakpoint locations (Figure 4A). We observed that the breakpoints were located on approximately opposite sides of the rod-like structure of the HDV RNA (Figure 4B), prompting us to examine the RNA structure of the JA-M36 genome. Indeed, JA-M36 was predicted to form an unbranched rod-like structure. The base-pairing pattern between the two breakpoints was demonstrated in Figure 4B.

Interestingly, one of the breakpoint identified in JA-M36 was found within the HDAg ORF, suggesting that the resulting chimeric HDAgs might be functional. Similarly, the recombinant RNA genome also provide an excellent experimental tool toward the understanding of the cis-elements crucial for HDV replication. Although replication-competent clones isolated from Miyako Island are not available, we have replication-competent HDV-1, HDV-2, and HDV-4 clones. These will be used as parental sequences to investigate HDV RNA recombination in cultured cells. Examination of the transactivation ability of the resulting chimeric small HDAgs and the replication activity of recombinant RNA genomes will contribute to the understanding of the molecular mechanism of HDV replication.

respectively. The sources of the HDV sequences are as mentioned in the legend to Figure 1. JA-M36 (white arrow) is the putative recombinant sequence and JA-M33 (dashed arrow) and JA-M11 (arrow) are its apparent parental sequences. Bootstrap resampling and reconstitution were carried out 1000 times. The numbers at the nodes indicate the bootstrapping values. The bar represents nt substitutions per position.

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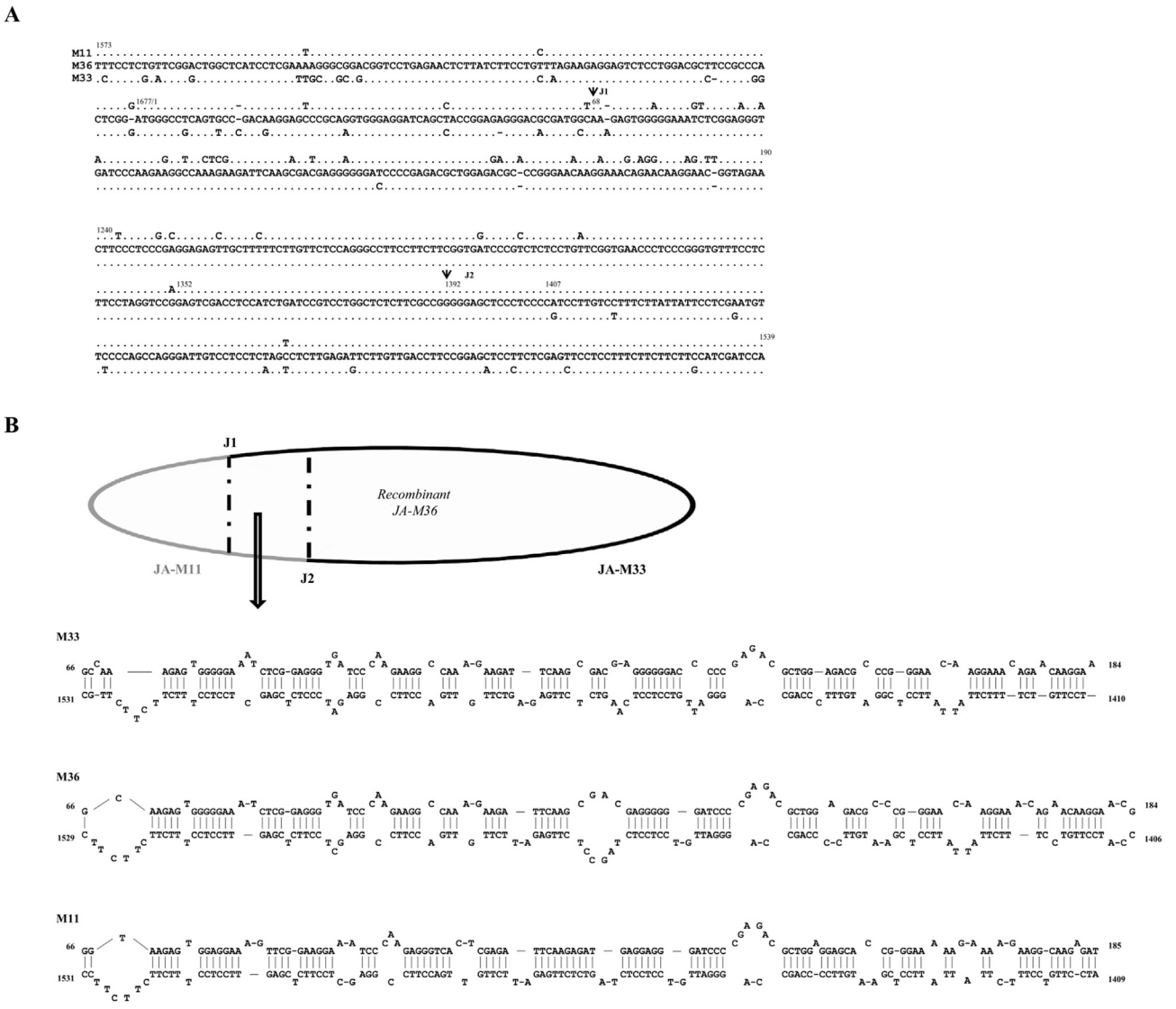

Figure 4. Analyses of the breakpoints and RNA structure of the novel recombinant, JA-M36: (A) alignments of Hepatitis delta virus sequences surrounding the breakpoints. The conserved nucleotides are denoted as dots. Two possible recombination junctions are shown by arrows; (B) diagrammatic representation of the base-pairing pattern through a portion of the recombinant Hepatitis delta virus genome. The rod-like structure of the recombinant JA-M36 RNA, labeled with two breakpoints J1 and J2, is shown at the top. The potential major (JA-M33) and minor (JA-M11) parental sequences are depicted as black and gray lines, respectively. Each side of the domain formed between the two dashed lines was derived from different parental sequences. The base-pairing pattern of this domain is shown at the bottom. The vertical lines indicate the base pairing formed by the circular RNA. The RNA structure was predicted using RNAstructure version 3.7.[30]

To date, fewer than 250 full-length HDV sequences have been published. This is a relatively small dataset compared with the samples used to perform phylogenetic analyses of recombination in other RNA viruses, such as the 1278 full-length genomic sequences studied for the hepatitis C virus (HCV)[36] and the 2197 IGSP (National Institute of Health Influenza Genome Sequencing Project) sequences studied for influenza viruses.[37] Notably, only two of 2197 genomic sequences of influenza virus and 14 of 1278 genomic sequences of HCV yielded recombination signals. HCV is a positive-sense RNA virus, whereas influenza virus and HDV are segmented and nonsegmented negative-sense RNA viruses, respectively. Homologous recombination is generally thought to be rare in negative-sense RNA viruses, perhaps reflecting that the ribonucleoprotein complex never disassembles from the RNA.[38] By contrast, we herein report that HDV recombination is not rare (1 recombinant out of 34 sequences analyzed) when the analyzed sequences have been prescreened to fit the above-mentioned criteria. Recombinant lineages have also been identified for HCV and Ebola virus, which is a nonsegmented negative-sense RNA virus.[39] In the future, a large-scale study involving molecular epidemiology could lead to the identification of a circulating clade of recombinant HDVs; this would provide far more compelling evidence that homologous recombination does, indeed, occur in HDV.

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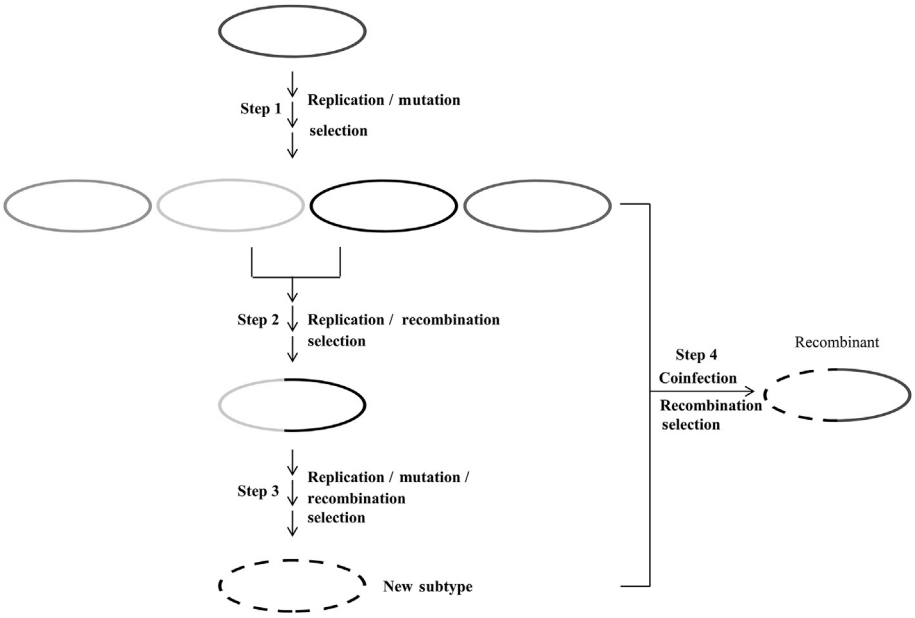

Figure 5. Schematic of a model for the evolution of genetic diversity in Hepatitis delta virus. The individual steps are described in the text. Ovals drawn with black, gray, and broken lines represent Hepatitis delta virus sequences with different numbers of mutations at different positions.

Chronic HDV infection is relatively rare in Japan, whereas HBV infection is prevalent. However, HDV is endemic in Miyako Island. This island, which is among the southwest islands of Okinawa, is located 1200 miles southwest of Tokyo and 500 miles northeast of Taiwan. Historically, there has been a trading relationship between the residents of Miyako Island and Taiwan since the 19th century.[22] Taiwan was occupied by Japan during World War II, and some people living on Miyako moved to Taiwan during this period.[22] The Miyako-specific HDV-4M subtype (82.4%) is predominant in Miyako Island, where HDV-2 (2.9%) and HDV-4 (14.7%) are also found.[15,21,22] By contrast, HDV-1, HDV-2, and HDV-4 (but not HDV-4M) have been isolated in Taiwan, where HDV-2 is the predominant genotype.[16] It has been noted that the single HDV-2 isolated from Miyako Island clustered with a Taiwanese clone, and the five HDV-4 sequences isolated from Miyako Island clustered with the reported Taiwanese HDV-4 sequences.[21] In Taiwan, HDV-2 is generally associated with a more favorable clinical outcome than HDV-1.[16] By contrast, patients on Miyako Island with HDV-4M showed greater progression to chronic hepatitis and cirrhosis than those with HDV-4.[15] The existence of a genetic subgroup (i.e., HDV-4M) that apparently has different clinical characteristics could point to genetic variations in functionally important parts of the HDV genome.

Mutations and homologous recombination, followed in all cases by selection, might contribute to the evolution of HDV. A four-step model for the evolution of genetic diversity in HDV is summarized in Figure 5. In the first step, mutations accumulate during replication. In the second step, homologous recombination between mutated RNA genomes could both remove harmful mutations and facilitate the accumulation of beneficial ones. This process would enable a far wider exploration of sequence diversity than can be achieved by mutation alone. In the third step, a series of mutations, homologous recombinations, and selections yield a new HDV subtype. Finally (the forth step and newly proposed in the present work), a mixed-subtype infection (such as HDV-4/HDV-4M) could generate recombinant HDV.

The prevalence of HDV infection has significantly declined in some regions of the world including Taiwan. However, the number of infected patients stopped decreasing towards the end of the 1990s in Europe, mainly because of an increased immigration from HDV endemic regions including Africa and Turkey.[4] Consequently, coexistence of multiple clades of HDV in Europe, similar to that observed in Miyako Island, will increase the occurrence of mixed-genotype infections of HDV and promote the generation of recombinant HDV.

HDV-1/HDV-2 recombinant in Vietnam

HDV is endemic in Vietnam. Based on 21 partial HDV sequences identified from Vietnamese patients, HDV-1 is the predominant HDV genotype (90.5%), and the rest belonged to HDV-2. However, only five complete sequences of Vietnamese origin have been reported, one of which appears to be an inter-genotypic HDV-1/HDV-2 recombinant.[40] This recombinant grouped with HDV-1 and HDV-2 based on phylogenetic analyses of regions covering nt 316—691 and corresponding to HDAg ORF, respectively, and reportedly had a breakpoint at nt 908 [using M92448 (HDV-1, Taiwan-1) and AF425645 (HDV-2) as parental sequences].[40] Since the authors of the previous publication did not use segments in total covering the whole genome for their phylogenetic analyses, and an HDV recombinant with only one breakpoint could not form a viable circular genome, we re-evaluated the Vietnamese sequences using the four-subdomain strategy presented in Figure 2. As shown in Figure 6, our

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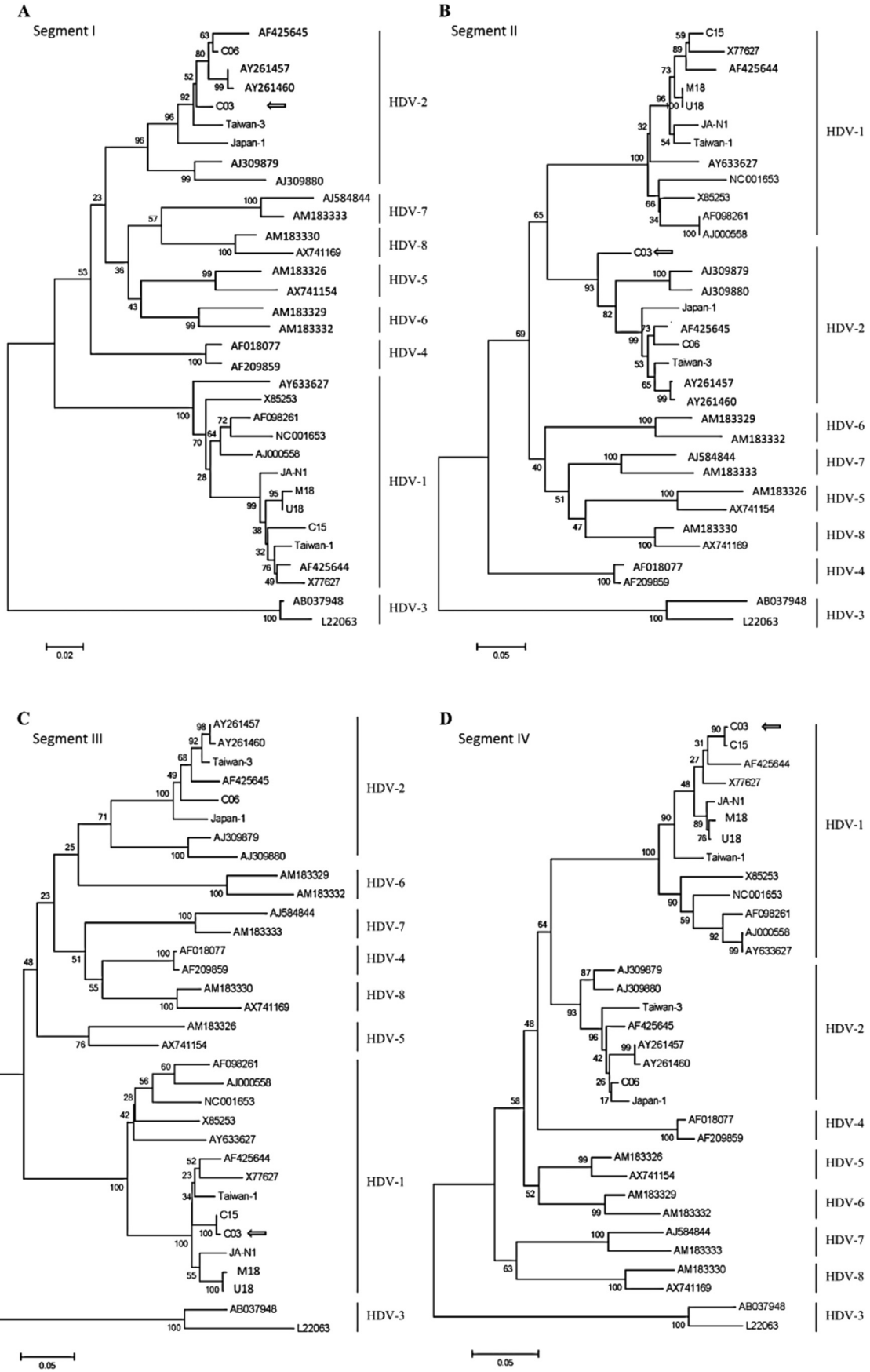

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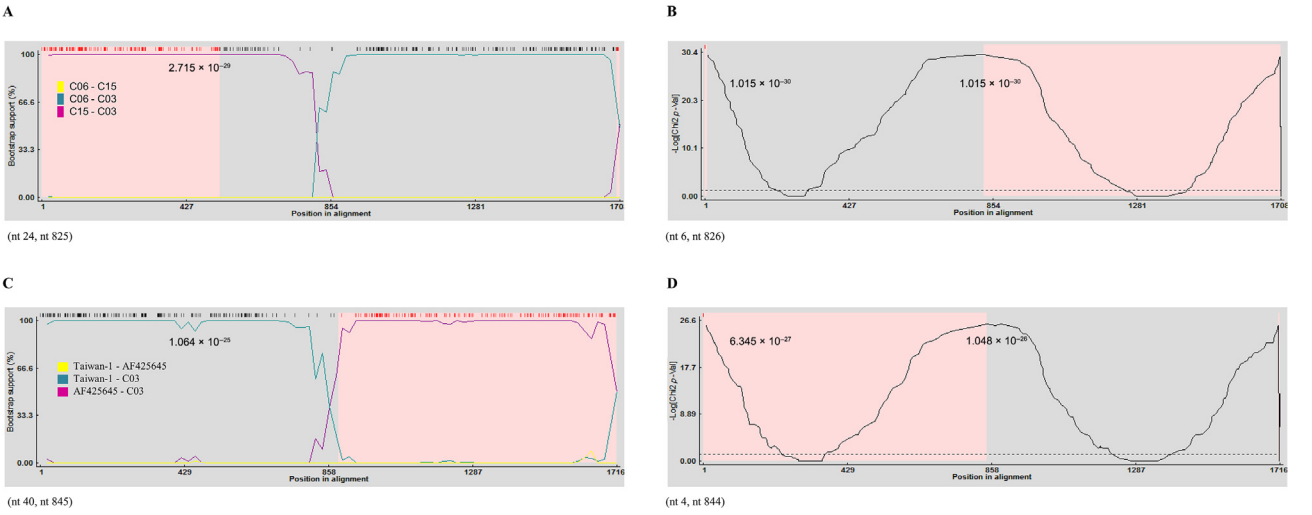

Figure 7. Analysis: (A) BootScan; (B) 3Seq; (C) Chimera; and (D) MaxChi recombination analyses of C03 and its potential parents. The y-axis gives the percentage of permuted trees in a sliding 100-bp window with 20-bp steps, as analyzed in the previous report.[40] The potential parental sequences, *p* values, and breakpoints are shown. Nt = nucleotides.

NJ phylogenetic analyses demonstrated the presence of two potential recombination events in this Vietnamese recombinant. The phylogenetic trees for segments III and IV showed that C03 clustered into HDV-1 with C15 as the closest sequence (Figures 6C and 6D). However, C03 clustered into HDV-2 when Segments I and II were subjected to analysis (Figures 6A and 6B). Significant sequence homology (> 99%) was observed between recombinant C03 and parental C15 in the region covering nt 1—849. By contrast, the sequence of C03 was highly homologous to that of C06 (97.8%) in the region spanning nt 850—1675. Although the parental sequences suggested here were different from the previously reported ones (M92448 and AF425645),[40] these data were consistent with the proposal that C03 is an HDV-1/HDV-2 recombinant. BootScan and Chimera recombination analyses of C03 with C15 and C06 were performed in parallel with those of C03 with M92448 and AF425645. Although two recombination junctions were observed in all cases, the locations of the breakpoints in one analysis were not exactly the same in other recombination analyses (Figure 7). Furthermore, the location of the two breakpoints in this HDV-1/HDV-2 recombinant did not favor the formation of an unbranched rod-like RNA structure. Therefore, this HDV-1/HDV-2 recombinant has not yet been fully substantiated by bioinformatic evidence.

Collectively, a number of criteria must be employed to minimize false-positive recombination signals and confirm the occurrence of homologous recombination in the history of a set of HDV sequences. Firstly, consistent data showing statistically significant recombination signals should be obtained using multiple independent phylogenetic and recombination analyses. Secondly, the unbranched rod-like RNA structure, which is essential for HDV replication, should be examined to further support the contention that the HDV recombinant has both a survival advantage and biological significance. Furthermore, the putative recombination events should be plausible as the most likely hypothesis for HDV RNA recombination. Since the HDV genome is circular and its replication occurs via a rolling-circle mechanism, two (or an even number of) crossovers should be identified in HDV recombinants.[19] If only one recombination event (or an odd number of events) occurs in the replicated HDV genome, the recombinant RNA will contain two junctions, one corresponding to the real recombination site and one representing the self-cleavage site. Conversely, if another recombination event occurs prior to the appearance of the self-cleavage site, both junctions between the two parental sequences will represent real recombination sites (as observed for the HDV-4/HDV-4M recombinant identified in this report). The patterns of recombination observed in this report displayed strong evidence that selection favors the survival of recombinant genomes with no disruption of the base-pairing interactions in the viral RNA structures.

Conflicts of interest

The authors have nothing to disclose.

Acknowledgments

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Figure 6. Recombination of Vietnamese sequences: (A—D) neighbor-joining phylogenetic trees of the four HDV sequence subdomains described in the legend to Figure 2. The Vietnamese sequences were compared to Hepatitis delta virus (HDV) reference sequences representing HDV-1—HDV-8 (denoted on the right) as described previously.[40] The white arrow represents the potential recombinant C03.[40] Bootstrap resampling and reconstitution were carried out 1000 times. The numbers at the nodes indicate bootstrapping values. The bar represents nucleotide substitutions per position. HDV = Hepatitis delta virus.

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